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Mouse Hepatitis Virus Infection Suppresses Modulation of Mouse Spleen T-cell Activation

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by

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Abbreviations: Con A, concanavalin A; LPS, lipopolysaccharide; MHV, mouse hepatitis virus; VAF, virus/antibody free.

Summary

Natural infection by mouse hepatitis virus (MHV) can affect interpretation of immunological studies in mice. MHV, a collective term describing a group of corona viruses (1-3), is found in natural infections in over 70% of laboratory mouse populations in the United States and Canada (4-6). Natural outbreaks of mouse hepatitis virus (MHV) in our animal colony afforded us the opportunity to study MHV-induced immunosuppression as well as the effects of MHV infection on neurotransmitter-immunomodulation. Concanavalin A-stimulated DNA synthesis by spleen T-lymphocytes from MHV-infected mice was 20-50% that of noninfected mice. The MHV infection also altered neurotransmitter modulation of spleen T-lymphocyte activation. In contrast to norepinephrine ablation of Con A-activated DNA synthesis by spleen lymphocytes from non-infected mice, DNA synthesis by the infected group was not inhibited by norepinephrine or dibutyryl-cAMP. These effects of MHV-infection were specific for spleen T-lymphocytes since MHV infection did not alter concanavalin A stimulation of thymocytes, lipopolysaccharide stimulation of spleen B-lymphocytes, or norepinephrine inhibition of thymocyte and B-cell DNA synthesis. MHV infection also did not alter spleen T-lymphocyte subset proportions. Thus, MHV infection inhibits spleen T-lymphocyte activation and blocks in vitro catecholamine and cAMP regulation of spleen T-cell activation but does not affect activation of thymic cells or spleen B-cells.

Introduction

Mouse hepatitis virus (MHV) is a common infection in laboratory mouse populations (4-6). The pathological effects of an infection with MHV varies as a function of a number of factors which include the strain of the virus, the route of infection, and the age and strain of the mouse (1, 2, 7). The effects of natural MHV infection on immune function have been little studied. It has been observed that laboratory-adapted strains of MHV can inhibit spleen T-cell DNA synthesis (8) but few investigations have studied effects of natural infections on immune responses or viral effects on neuroimmunomodulation.

Most recently, this laboratory has been examining immunomodulation by neurotransmitters. Neurotransmitter regulation of immune responses is physiologically significant since lymphoid organs are innervated (9), lymphoid tissue contains neurotransmitters (9), and neurotransmitters have been shown to regulate immune responses both *in vivo* and *in vitro* (9-14). The recent studies in our laboratory of the mechanism(s) of neurotransmitter modulation of the immune system showed that norepinephrine and related catecholamines can negatively modulate mitogen-induced T-cell and B-cell DNA synthesis in a serum-free *in vitro* system (15). Surprisingly, these studies revealed that the pathway(s) of inhibition by norepinephrine may differ in T-cells and B-cells (15).

In the work reported here, we examined the effects of natural

MHV infection on mitogen-stimulated DNA synthesis and its regulation by norepinephrine in mouse splenic T-cells, splenic B-cells and thymic cells cultured under serum-free conditions.

Materials and Methods

Animals. Male BALB/c mice (4-6 weeks old) were obtained from Harlan, Indianapolis, IN and husbanded in the Biologic Resource Laboratory, an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved animal care facility, in accordance with institutional guidelines. Virus/antibody free (VAF) mice were naturally infected by periodic outbreaks of MHV at our institution.

Murine Hepatitis Analysis. MHV infections were determined by analyzing serum from randomly sampled BALB/c male mice (6-10 weeks old) in the colony by an ELISA (Charles Rivers Laboratories, Bloomington, MA) for anti-MHV antibodies. Since MHV is a highly contagious virus, all cage-mates of the infected mice were assumed to be infected.

Cell Culture of Splenic and Thymic Cells. Cells were cultured in serum and protein free culture medium consisting of RPMI-1640 (with glutamine, Gibco, Grand Island, NY) supplemented with 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Mice were sacrificed by cervical dislocation and their spleen or thymus was excised. At room temperature, the tissue was minced and gently pressed through a sterile Nytex nylon mesh (Tetko, Inc., Elmsford, NY) with a sterile stainless steel lab spoon (American Scientific Products, McGaw Park, IL). The resulting single cell suspension was washed twice with medium. Cell viability at culture initiation, determined by trypan blue exclusion (16), was >85%. Cells (100 μl of 5 x 10^6 cells/ml) were placed in a 96 well flat bottom plate and incubated

at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Mitogenic Stimulation of DNA Synthesis. Splenic or thymic cell cultures were stimulated with concanavalin A (Con A, Pharmacia, Piscataway, NJ) or lipopolysaccharide (LPS phenol extract from E. coli 055:B5, Sigma). These mitogens were used at concentrations that gave maximal stimulation of DNA synthesis. Norepinephrine (Sigma) or N^6,2'-O-dibutyryl cyclic adenosine monophosphate (Sigma) were added immediately before mitogenic addition. The norepinephrine (100 pM to 100 μM) was added in dilute HCl. At these concentrations (≤100 μM), HCl did not effect DNA synthesis by resting or mitogen stimulated cells. After 44 hours of incubation, 1 μCi of [methyl-^{3}H]thymidine (6.7 μCi/mmole, DuPont Co., N. Billerica, MA) was added to each well. Four hours later the cultures were harvested onto glass fiber filters (Grade 934AH, Whitaker, M.A. Bioproducts, Walkersville, MD) and washed 20 times with distilled water using a multiple sample harvester (Whitaker). The amount of [methyl-^{3}H]thymidine incorporated into DNA was measured by liquid scintillation counting. Nonstimulated cells incorporated less than 3000 cpm [methyl-^{3}H]thymidine. At the time of harvest, cell viability, determined by trypan blue exclusion or fluorescein diacetate fluorescence (16), was always >65% or >55%, respectively. Norepinephrine and dibutyryl cAMP did not affect cell viability.

Analysis of T-cell Subpopulations. A single cell suspension of spleen lymphocytes were placed over a lympholyte M gradient (Accurate Chemical and Scientific Corp.)and the lymphocytes at the

interface of the gradient were collected and washed tree times with 0.1M phosphate buffered saline (PBS, pH7.4) solution containing 0.15% bovine serum albumin (tissue culture grade BSA, Sigma , St. Louis, MO) and 0.15% NaN_3. Spleen cells (10^6) were placed on ice and resuspended in the above solution prior to addition of 5μl of fluorescein isothiocyanate (FITC) conjugated affinity purified rat anti-mouse Lyt2 (Becton Dickenson, Mountain View, CA) and/or phycoerythrin conjugated affinity purified rat anti-mouse L3T4. After 30 minutes, the cells were washed 3 times and analyzed by flow cytometry.

Statistics. Comparison of one value to a control value was evaluated by the Student's T-test. Comparison of more than one value to a control value was evaluated by a completely random ANOVA followed by Dunnett's Test. Values were considered significantly different at $p<0.05$.

Results

Effects of Natural MHV Infections on Spleen T-cell, Spleen B-cell and Thymus Cell DNA Synthesis. Spleen and thymus cell DNA synthesis was stimulated by concanavalin A (Con-A), a T-cell specific mitogen, or by lipopolysaccharide (LPS), a T-cell-independent B-cell specific mitogen. Natural infection with MHV dramatically reduced (50-80%) Con-A-stimulated DNA synthesis by spleen lymphocytes (Figs. 1 and 2); optimal stimulatory concentrations of Con-A (0.25μg - 0.5μg/ml) were the same for both groups of mice (Fig. 1). In contrast, Con-A-stimulated thymocytes or LPS-stimulated spleen lymphocytes revealed no differences between cells from infected and noninfected mice. Con-A stimulated the incorporation of 30844 ± 1028 cpm [^{3}H]thymidine by thymus cells from MHV infected mice and 24494 ± 2354 cpm [^{3}H]thymidine by thymus cells from noninfected mice in a representative experiment from three experiments. LPS stimulated the incorporation of 60136 ± 7908 cpm [^{3}H]thymidine by spleen cells from MHV infected mice (mean ± SEM of 12 experiments) and 65390 ± 5472 cpm [^{3}H]thymidine by spleen cells from noninfected mice (mean ± SEM of 22 experiments). Thus, natural MHV infection appears to specifically inhibit the activation of spleen T-cells but not spleen B-cells or thymocytes.

Effects of Natural MHV Infections on Norepinephrine Inhibition of Con A-Stimulated Spleen Cell DNA Synthesis. Norepinephrine is thought to play a role in the regulation of the immune system (9, 10, 13). We previously reported that norepinephrine inhibits mitogen activation of murine T-cells and B-cells (15).

Norepinephrine (100μM) completely inhibited the 50 fold Con-A stimulation of DNA synthesis by spleen lymphocytes from noninfected mice (Fig. 2). In contrast, the six fold stimulation of DNA synthesis by spleen T-cells from MHV-infected mice was unaffected by norepinephrine (Fig. 2). However, MHV infection did not impair the ability of norepinephrine to inhibit the activation of thymocytes by Con-A or the activation of spleen cells by LPS (Table 1). Thus, MHV infection blocked the neural-immune modulation of DNA synthesis in only spleen T-cells.

Effect of MHV Infection on Dibutyryl cAMP Inhibition of Con-A-stimulated DNA Synthesis by Spleen and Thymus Cells. Since dibutyryl cAMP is an analog of the norepinephrine second messenger cAMP (15), it was of interest to determine if MHV infection could affect dibutyryl cAMP inhibition of DNA synthesis in ways similar to MHV effects on norepinephrine inhibition. Dibutyryl cAMP did not inhibit Con-A-stimulated DNA synthesis in spleen lymphocytes from MHV-infected mice but did retain its capability to inhibit Con-A-activation of thymocytes from MHV-infected mice (Fig. 3). Thus, the loss of norepinephrine responsiveness by spleen T-lymphocytes in MHV-infected animals is associated with a parallel resistance to cAMP action.

Effect of MHV Infection on Numbers and Classes of Lymphocyte Subpopulations. Disorders of immune regulation are often accompanied by alterations in the numbers and classes of lymphocyte subpopulations. In the natural MHV infection, no differences were found in the number of lymphocytes per spleen as compared to

noninfected mice (data not shown). Furthermore, MHV infection did not affect the proportion of T-cell types. Spleens from MHV-infected and noninfected mice contained 0.6-0.8% $Lyt2^{+}L3T4^{+}$, 31-32% $Lyt2^{+}L3T4^{-}$, and 12-14% $Lyt2^{-}L3T4^{+}$ T-cells as determined by two-color flow cytometry (Table 2). However, flow cytometry analysis of the T-cell types revealed a significant increase (13-32%) in number of larger $Lyt2^{+}L3T4^{-}$ and $Lyt2^{-}L3T4^{+}$ spleen lymphocytes from infected mice as compared to that from noninfected mice (data not shown). The size of these larger cells from MHV-infected mouse spleen was similar to that of large blast lymphocytes from mitogen-stimulated cultures.

Discussion

Natural MHV infection altered DNA synthesis of specific lymphocyte populations. The MHV-infection reduced DNA synthesis by Con A-stimulated spleen cells but not LPS-stimulated spleen cells or Con A-stimulated thymus cells. Natural MHV infection also blocked neurotransmitter modulation of DNA synthesis by specific lymphocyte populations. In contrast to norepinephrine inhibition of Con A-stimulated DNA synthesis by spleen T-cells from non-infected mice, norepinephrine did not inhibit DNA synthesis by spleen T-cells from MHV-infected mice. Neuromodulation of spleen B-cells or thymus cells was not altered by the infection since norepinephrine inhibited DNA synthesis by spleen B-cells or thymus cells from MHV-infected and non-infected mice. The concentrations of norepinephrine used in this study are physiologically relevant, since the K_D for the binding of norepinephrine to β-adrenergic receptors on lymphocytes is approximately 100 μM (17, 18). Interstitial concentrations of norepinephrine in the spleens of non-stressed mice are reported to be in the range of 1μM, but higher concentrations may be present in the restricted spaces between neurons and lymphocytes (9). The lack of effects of norepinephrine on spleen T-cells from MHV-infected mice were paralleled by a similar nonresponse to dibutyryl cAMP, an analog of cAMP which is the second messenger for norepinephrine signal transduction in T-cells (15). This finding suggests that MHV infections might interfere with norepinephrine action at a step subsequent to norepinephrine receptor binding and adenylyl cyclase

activation in spleen T-cells.

The reduced DNA synthesis by spleen T-cells from MHV-infected mice was not a result of a loss in number of T-cells or a change in proportion of T-cells in the spleen since the MHV-infection did not alter the percentage of T-cell subpopulations in the spleen. The percentage of $CD4^+$ T-cells in the spleens of mice infected with MHV-JHM, a laboratory adapted strain of MHV, was also not altered by MHV infection (Abigail Smith, personal communication). Although the MHV infection did not affect the proportion of T-cell subpopulations, the natural infection increased the number of large blast-like T-cells in the spleen. The relationship between the increased number of larger lymphocytes and altered lymphocyte responses to Con-A is unknown. Taken together, the results suggest that reduced DNA synthesis by spleen T-cells from MHV-infected mice may be a result of an aberrant spleen T-cell function(s).

Smith et. al. (8) reported that BALB/cByJ mice infected with MHV-JHM, a laboratory adapted strain of MHV, showed reduced DNA synthesis and IL-2 production in response to Con-A. Our findings indicate that natural MHV infection not only specifically affects the activation of spleen T-cells but also blocks their response to norepinephrine. The decreased T-cell response to Con-A is unlikely to be secondary to changes in macrophage function, because our cultures contained 2-mercaptoethanol, which can replace the macrophage requirement for lymphocyte activation (19) and Smith et. al. (8) have reported that MHV-JHM infection does not affect IL-1 production by macrophages.

The type of lymphocyte in which MHV replicates is dependent upon the strain of MHV. For example, the MHV-JHM strain replicates in macrophages and B-cells (A. Smith, personal communication) whereas the MHV3 strain replicates lytically in B-cells and T-cells (20). In contrast to laboratory adapted strains of MHV, there are a great number of natural strains of MHV and, for many, specific identification assays are not available. Further, the natural MHV strains are capable of mutating. These difficulties make the identification of the specific MHV strains in natural MHV infection problematic. Thus, no attempt was made in these studies to characterize the sub-strains of MHV present in the natural viral murine infection. The relationship between viral replication in the natural infections and the altered spleen T-cell responses to Con-A and norepinephrine is yet to be understood.

Laboratory mouse populations are commonly infected by MHV (4-6). The finding that MHV infection depresses spleen T-cell activation before anti-MHV antibodies can be detected by serology (8) highlights both the importance and the difficulty of excluding MHV infection in immunological studies. Analysis of the mechanism(s) by which MHV affects lymphocyte activation may yield insights into viral-mediated immunosuppression.

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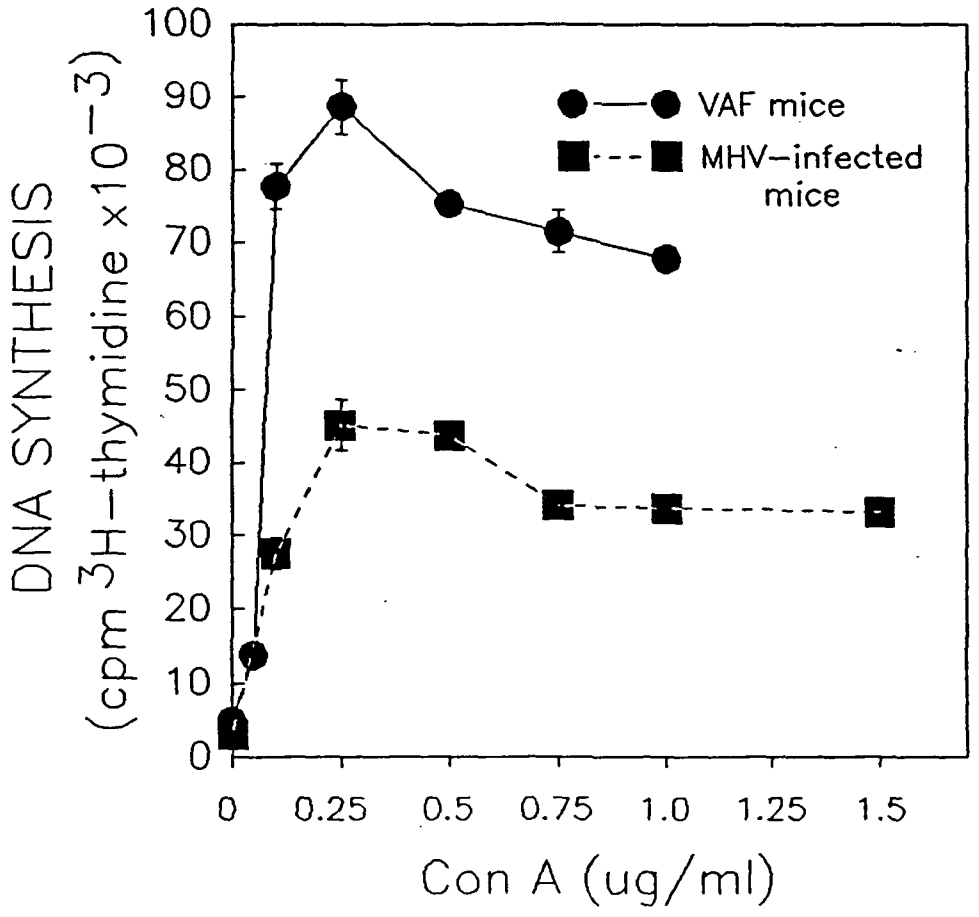
Figure Legends

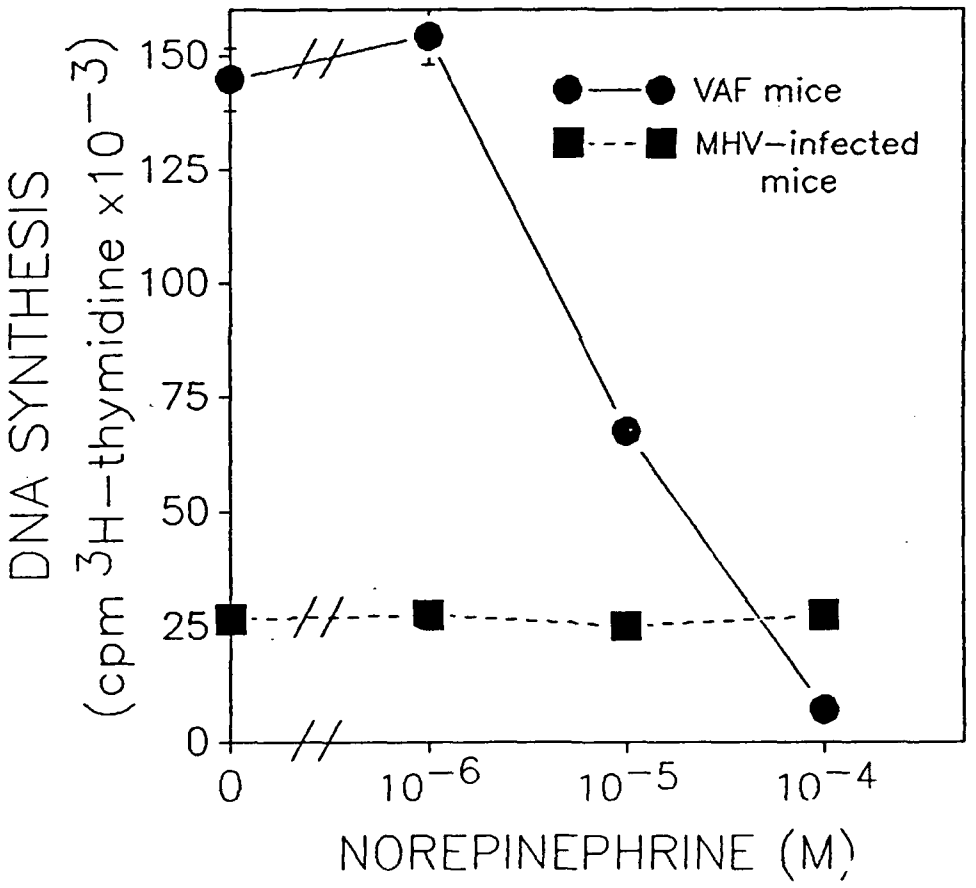
Fig. 1 Con A-stimulated DNA synthesis by spleen cells from MHV-infected and virus/antibody free (VAF) mice. Spleen cells were cultured in the presence of Con A. After 44 hours, DNA synthesis was determined by addition of 1 μCi [methyl-^{3}H]-thymidine for 4 hours followed by harvesting and liquid scintillation counting. Data are presented as mean ± SEM from quintuplicates of a representative experiment. All values for the MHV-infected mice were significantly different ($p<0.001$) than those for the VAF mice as determined by the Student's t-test. Non-stimulated cells incorporated less than 3000 cpm [methyl-^{3}H]-thymidine (6.7 Ci/mmole, DuPont, N. Billerica, MA).

Fig. 2 Norepinephrine regulation of Con A-stimulated DNA synthesis by spleen cells from MHV-infected and Virus/antibody free (VAF) mice. Spleen cells from VAF and MHV-infected mice were cultured and DNA synthesis was measured as described in Fig. 1. Norepinephrine was added to the cells immediately before addition of Con A (0.5 μg/ml). Non-stimulated cells incorporated less than 3000 cpm [^{3}H]-thymidine. Data are presented as mean ± SEM from a representative experiment. All values for the MHV-infected mice were significantly different ($p<0.001$) than those for the VAF mice as determined by the

Student's T-test.

Fig. 3 Effect of Dibutyryl cAMP on Con A-stimulated DNA synthesis by spleen and thymus cells from MHV-infected and Virus/antibody free (VAF) mice. Dibutyryl cAMP (5×10^{-4}M) was added immediately before addition of Con A (0.5 μg/ml). Cells were cultured and DNA synthesis was determined as described in Fig. 1. It was previously determined that 5×10^{-4}M dibutyryl cAMP maximally inhibited Con A-stimulated DNA synthesis (21). The % of Con A-stimulated control is presented as mean ± SEM from an experiment for the spleen cells and, for the thymus cells, from the same representative experiment shown in Fig. 2. Asterisk indicates a significant difference ($p<0.05$) from the Con A-stimulated control for each group as determined by the Student's t-test.


DNA SYNTHESIS
(cpm ^{3}H–thymidine x10^{-3})
100
90
80
70
60
50
40
30
20
10
0
0
0.25
0.5
0.75
1.0
1.25
1.5
Con A (ug/ml)
VAF mice
MHV–infected mice


DNA SYNTHESIS
(cpm ^{3}H-thymidine x10^{-3})
150
125
100
75
50
25
0
VAF mice
MHV-infected mice
0
10^{-6}
10^{-5}
10^{-4}
NOREPINEPHRINE (M)

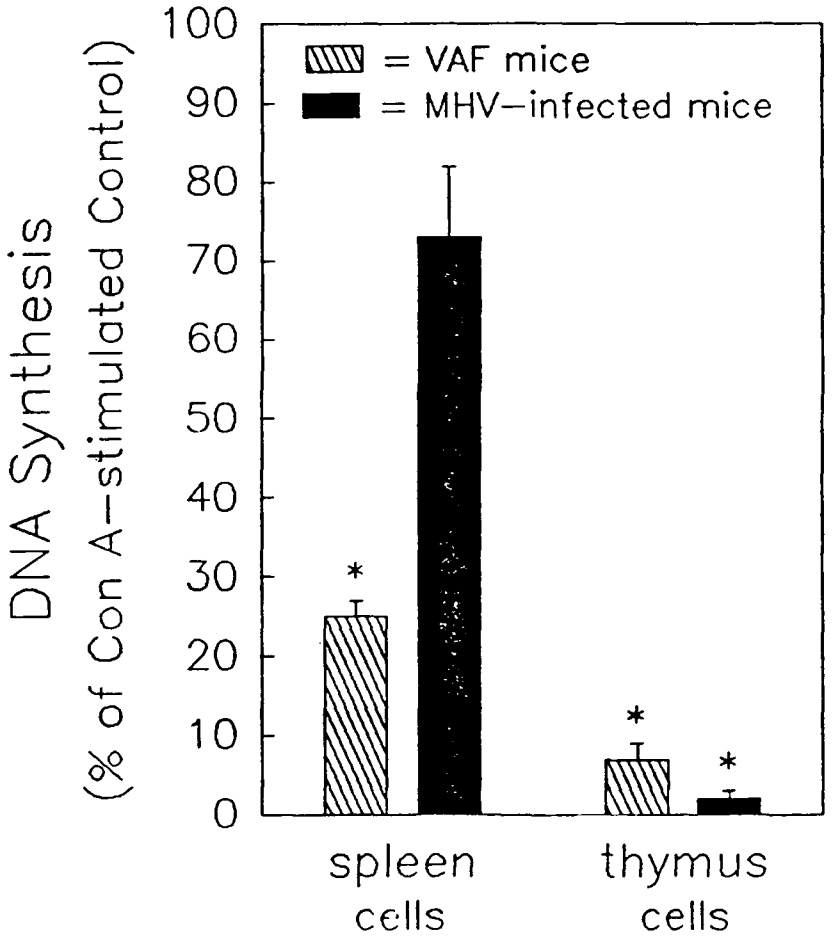

DNA Synthesis
(% of Con A–stimulated Control)
100
90
80
70
60
50
40
30
20
10
0
= VAF mice
= MHV–infected mice
*
*
*
spleen cells
thymus cells

Table 1 Effects of Norepinephrine on Con A-stimulated DNA Synthesis by Thymus Cells and LPS-stimulated DNA Synthesis by Spleen Cells from MHV-infected and Viral/antibody free (VAF) Mice.

		DNA Synthesis[a] (% of mitogen-stimulated control)	
		VAF Mice	MHV-infected Mice
LPS-stimulated spleen cells[b]	NE(10μM)	56 ± 3	47 ± 4
	NE(50μM)	62 ± 5	50 ± 7
	NE(100μM)	21 ± 6	32 ± 8
ConA-stimulated thymus cells[c]	NE(50μM)	72 ± 7	82 ± 4
	NE(100μM)	38 ± 5	3 ± 1

[a] Thymus cells and spleen cells from VAF and MHV-infected mice were cultured and DNA synthesis was measured as described in Fig. 1. Norepinephrine (NE) in 10^{-4} to 10^{-6}M HCl was added to the cells immediately before addition of Con A (0.5μg/ml) or LPS (12μg/ml). HCl at $\leq 10^{-4}$M did not affect DNA synthesis by non-stimulated or mitogen stimulated cells. Non-stimulated cells incorporated less than 3000 cpm [^{3}H]-thymidine (i.e. 3-7% of mitogen-stimulated control DNA synthesis).

[b] The % of LPS-stimulated control DNA synthesis is presented as mean ± SEM from 8 experiments for the MHV-infected mice and 18 experiments for the VAF mice. There is no significant difference ($p<0.05$) between the MHV-

infected and VAF groups as determined by the Student's t-test. Values are significantly lower ($p<0.05$) than that for the LPS-stimulated control as determined by a completely random ANOVA followed by Dunnett's Test.

[c] Data are presented as mean ± SEM from a representative experiment. Values are significantly lower ($p<0.05$) than that for the Con A-stimulated control as determined by a completely random Anova followed by Dunnett's Test.

Table 2. Proportion of T-cell Subsets in Spleen from MHV-infected and Viral/antibody free (VAF) Mice.

T-cell subset	% Fluorescent Cells[a] VAF mice	MHV infected mice
Lyt2 + L3T4	0.6 ± 0.1[b]	0.8 ± 0.2
L3T4	31.9 ± 0.6	31.3 ± 0.9
Lyt2	14.4 ± 1.0	12.2 ± 0.6

[a] Spleen cells from MHV-infected or VAF mice were incubated with fluorescein-conjugated anti-Lyt2 and/or rhodamine-conjugated anti-L3T4 in PBS/0.15% BSA/0.15% NaN_3 at 4°C for 30 minutes. The cells were washed 3 times and T-cell subsets were determined by two-color flow cytometry.

[b] mean ± SEM of 3 experiments.